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Effects of Mycoplasma gallisepticum on experimentally infected Eastern Bluebirds (Sialia sialis)

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Effects of *Mycoplasma gallisepticum* on experimentally infected Eastern Bluebirds (*Sialia sialis*)

by
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ABSTRACT

The bacterium *Mycoplasma gallisepticum* is an avian respiratory pathogen that causes inflammation and swelling of conjunctiva in domestic poultry and wild finches. In the past, severity of conjunctivitis symptoms has been used to quantify infection severity and host resistance. However, many songbirds function as hosts to *M. gallisepticum* but do not develop this symptom at all. The absence of conjunctivitis, as well as other clinical signs, hinders our understanding of *M. gallisepticum* infection in the songbird community because some species are responding very differently than others. One such species is the Eastern Bluebird (*Sialia sialis*), a common songbird of the southeastern United States. In efforts to determine if *M. gallisepticum* is a respiratory pathogen of Eastern Bluebirds, data and samples collected from an experimental infection of wild-caught, captive housed birds were used. We quantified relative bacterial load in the eye conjunctiva and choanal cleft palate of infected birds and found that *M. gallisepticum* did not localize in the conjunctiva but was present in the palate of six out of nine birds 13 days following experimental infection. At this same time point, infected birds showed no significant difference in body condition measures when compared to controls but had significantly lower levels of circulating hemoglobin than did controls. Among infected birds, individuals with the greatest number of *M. gallisepticum* in their palate 13 days following infection lost more mass than did those with fewer bacteria. Hemoglobin levels and other measures of condition were not significantly correlated with pathogen load. These results suggest that although Eastern Bluebirds do not develop conjunctivitis, they are suffering from physiological consequences of infection. Furthermore, this could be the case in other host species that also do not develop conjunctivitis.

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Introduction

The study of emerging infectious diseases is key to broadening our understanding of the health of human populations and wildlife communities. A primary mode of disease diagnosis is via traditional clinical sign examination. For example, most humans with chickenpox are easily diagnosed simply by visual examination rather than having any sort of laboratory test run (Freer & Pistello 2018). This method of diagnosis can be problematic with many diseases as the type and severity of signs and symptoms may vary among individuals within a species as well as among different host species (Dhondt et al. 2008, Dhondt et al. 2014). Furthermore, using clinical sign examination to diagnose disease is inadequate in the case of asymptomatic individuals as infected hosts lack clinical manifestations and may appear completely healthy (Laishram et al. 2012). While clinically asymptomatic individuals or species do not appear to be suffering from infection, they very well could be suffering from costly physiological consequences, similar to a symptomatic individual (Cheatsazan et al. 2013). Because of this, asymptomatic species can pass as unrecognized hosts.

Once an organism is infected by a pathogen, it can respond to the infection through passive tolerance or active immunological resistance to the pathogen (Råberg et al. 2008). There are costs associated with both ways of responding and host-pathogen relationships can vary greatly; thus, one method is not always superior. Mounting a strong immune response requires a significant amount of resources, and it is not always

beneficial to the host. When tolerating an infection, the host minimizes the damage done by the pathogen rather than trying to eliminate the pathogen. This can include repairing tissue damage done by the pathogen and up-regulating physiological mechanisms that normally maintain homeostasis to lessen the burden of being infected (Medzhitov et al. 2012). If the negative effects of being infected by a pathogen are too great, a host may resist infection by employing its immune system to actively ward off the pathogen (Råberg et al. 2008). Often when a host is combating infection, the clinical signs and symptoms we associate with being sick, such as inflammation, fever, or mucus production, are results of a host's immune response and not the pathogen itself (Chaplin 2010). Therefore, a host that is displaying resistance to a pathogen, in many cases, will be obviously symptomatic.

Mycoplasma is a genus of bacteria which includes species that are often obligate pathogens to animals or plants (Levisohn & Kleven 2000). Relationships between *Mycoplasma* species and their hosts have been of growing research interest as advancements have been made towards understanding *Mycoplasma* pathogenicity, or how they infect and persist within a host (Rottem 2003; Citti et al. 2010). *Mycoplasma* species are capable of antigen switching, meaning they can alter their surface proteins. This aids in avoiding detection by a host's immune system and is a likely reason these species may continue to persist within hosts that mount a strong immune response (Razin et al. 1998; Levisohn & Kleven 2000; Citti et al. 2010).

Pathogenic species within the genus *Mycoplasma* are commonly found in the mucosal membranes of the respiratory tract, urogenital tract, eyes, mammary glands, and joints of an infected host (Levisohn & Kleven 2000). Common symptoms of pathogenic

mycoplasmas, such as *M. pneumoniae,* include ocular manifestations such as conjunctivitis and uveitis, respiratory distress, and hemolytic anemia (Liu & Janigian 2013; Waites & Talkington 2004). Other symptoms associated with *Mycoplasma* infections involve irregular weight patterns, such as reductions in weight gain and carcass value in cattle infected with *M. bovis* (Rosengarten & Citti 1999).

Mycoplasma gallisepticum is an avian pathogen that causes chronic respiratory disease in chickens, and infectious sinusitis in turkeys (Kleven 2003). *Mycoplasma gallisepticum* can be transmitted vertically as well as horizontally (Kleven 2003; Nolan et al. 2004; Adelman et al. 2013). Horizontal transmission can occur from direct contact with an infected bird, or indirectly such as through contaminated food or water (Kleven 2003). Although a host can symptomatically recover from the disease, they often remain chronic carriers of *M. gallisepticum* and may continue to transmit it to others (Kleven 2003). Controlling *M. gallisepticum* infection is therefore difficult for poultry farmers that house many birds in a small space (Kleven 2003; Osman et al. 2009). Thus, *M. gallisepticum* is able to spread rapidly, and its emergence typically leads to culling of entire flocks (Evans et al. 2005).

Common symptoms seen in birds infected with *M. gallisepticum* include coughing, sneezing, nasal discharge, periorbital swelling, and conjunctivitis (Kleven 2003). Such conjunctivitis is associated with eyes that are red and puffy and ocular discharge. In some cases, the eyes swell until they are completely closed, rendering the host effectively blind (Kleven 2003). Conjunctivitis can be defined as infection and inflammation of the conjunctiva (Azari & Barney 2013). The conjunctiva is a thin mucous membrane that lines the sclera of the eye as well as the inside of the eyelids

(Azari & Barney 2013). Conjunctivitis associated with *M. gallisepticum* infection is not caused directly by the bacteria. Instead, it is the host's immune response to the pathogen that is resulting in swelling and redness of the tissue surrounding the eye.

In 1994, House Finches were found to be infected with *M. gallisepticum* (Fischer et al. 1997). As with chickens, conjunctivitis was the primary diagnostic symptom of *M. gallisepticum* infection. In House Finches, conjunctivitis develops approximately four days after inoculation (Kollias et al. 2004). Furthermore, *M. gallisepticum* was found to be localized in the conjunctiva of infected House Finches (Fischer et al. 1997). In a study of House Finches experimentally inoculated with *M. gallisepticum*, Vinkler et al. (2018) found the severity of conjunctivitis was correlated with conjunctival pathogen load as well as pro-inflammatory interleukin 1 beta signaling. This suggests that the harm done to House Finches infected with *M. gallisepticum* is at least in part due to the birds' pro-inflammatory immune response (Vinkler et al. 2018). In previous studies conducted on House Finches experimentally infected with *M. gallisepticum*, disease progression has been monitored using several different methods, including scoring eye symptoms. Eye scoring involves ranking the intensity of conjunctivitis for each bird and serves as a measure of symptom severity (Kollias et al. 2004; Balenger et al. 2015), which correlates with finch tolerance, resistance, and rate of recovery from infection with *M. gallisepticum* (Kollias et al. 2004; Hill & Farmer 2005; Adelman et al. 2013). In recent years it has become common to also quantify *M. gallisepticum* load, or the relative number of *M. gallisepticum* cells harbored in the eye conjunctiva of infected finches in order to evaluate individual and population-level resistance (Bonneaud et al. 2011; Hawley et al. 2012; Adelman et al. 2013).

Mycoplasma gallisepticum is now known to infect dozens of species of passerines (Dhondt et al. 2014; Farmer et al. 2005; Ley et al. 2016), including Eastern Bluebirds (Balenger, unpubl. data). Unlike House Finches, Eastern Bluebirds do not develop conjunctivitis in response to *M. gallisepticum* infection (Balenger, unpubl. data). Because Eastern Bluebirds do not develop conjunctivitis, they are not suffering from the negative effects associated with that swelling, such as impaired vision. Although Eastern Bluebirds do not develop conjunctivitis and appear asymptomatic, they do mount a strong humoral immune response when infected, developing antibodies to *M. gallisepticum* within 13 days of infection (Balenger, unpubl. data).

The first objective of this study was to determine if *M. gallisepticum* is causing a disease state in Eastern Bluebirds. We asked whether there were physiological costs associated with being experimentally infected with *M. gallisepticum*. We predicted that birds infected with *M. gallisepticum* would have lower mass and lower overall body condition compared to controls. If *M. gallisepticum* infection is causing hemolytic anemia, we also predicted that infection would result in relatively low levels of circulating hemoglobin. Furthermore, considering the absence of conjunctivitis, we aimed to determine where *M. gallisepticum* was localizing in infected birds. If it is present in the eye conjunctiva in the absence of conjunctivitis, this would be consistent with a tolerant phenotype in response to infection with *M. gallisepticum*. If *M. gallisepticum* is not present in the eye conjunctiva, but instead only found in the choanal cleft, this would suggest that the mucosal lining of the eyes of Eastern Bluebirds is a poor match or an undesirable environment for *M. gallisepticum* compared to that of species that do develop conjunctivitis. Finally, if infection is costly in some way, we asked if the relative amount

of *M. gallisepticum* in the conjunctiva or choanal cleft relates to those costs. If Eastern Bluebirds are rigorously resisting *M. gallisepticum* infection, we predicted that an increased pathogen load would be correlated with greater loss of mass and decreased body condition. If Eastern Bluebirds are tolerating the infection, we predicted that birds with greater pathogen loads would not lose mass or body condition relative to those with low pathogen loads. We also examined whether pathogen load affected circulating hemoglobin levels. Because hemolysis of red blood cells is a result of pathogenesis, not host immunity, we predicted that lower pathogen loads would correspond with relatively higher hemoglobin levels regardless of whether hosts are relatively resistant or tolerant (Messick 2004).

Methods

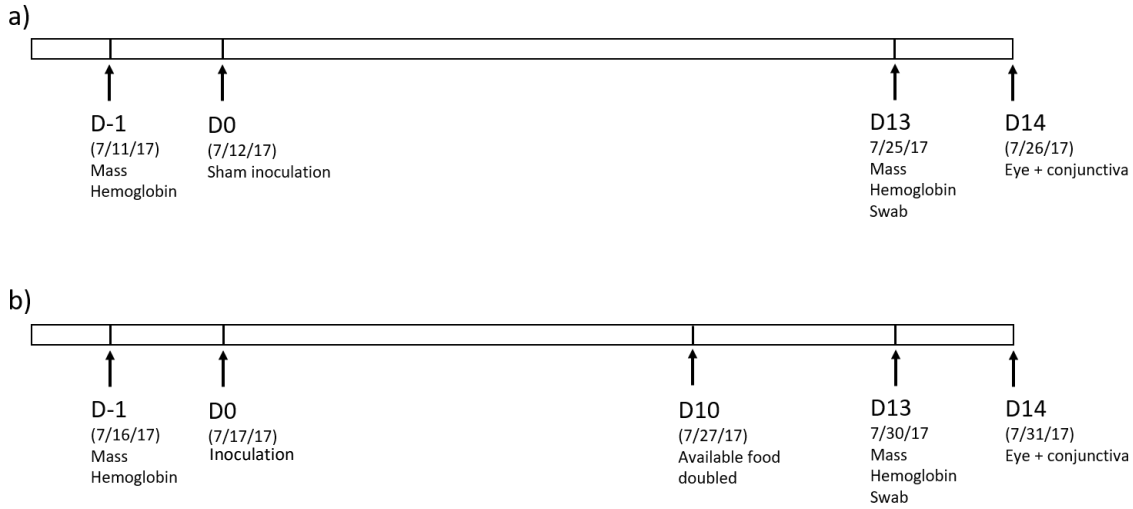
Sample sources

In this study, measures of body condition, individual mass, mass change, and hemoglobin levels as well as choanal cleft palate swabs and conjunctival samples collected from a previously done experimental infection were used. For use in the experimental infection, wild male and female adult Eastern Bluebirds were captured at the University of Mississippi Field Station (Abbeville, MS, USA) from April-June 2017. At the time of capture, standard measures of body condition were collected (mass and wing length), as well as samples to test for the presence of active or prior *M. gallisepticum* infection. To do so, the choanal cleft palate of each bird was swabbed and a small blood sample from the brachial wing vein was collected. Choanal swabs were tested for *M. gallisepticum* DNA via polymerase chain reaction (PCR) using species-specific primers for the cytadhesin gene *mgc2* (Roberts et al. 2001), and blood serum was tested for *M. gallisepticum* antibodies using a serum plate agglutination assay (SPA; Charles River Laboratories). Only birds that were confirmed to have had no prior exposure to *M. gallisepticum* were used. Birds that were found to be previously exposed were released back into the local populations. Subsequently, 24 birds (12 male, 12 female) were quarantined for inclusion in the experiment.

All birds were quarantined to individual cages (40’ x 20’ x 20’) at the University of Mississippi for a period of at least 30 days. After the quarantine period, all birds were retested for *M. gallisepticum* antibodies and presence of *M. gallisepticum* DNA from

blood and swab samples; one bird tested positive for antibodies at this time and was removed from the study. Beginning on the day of capture, birds were fed 30 g of mealworms (Fluker Farms) per day. This amount was roughly equivalent to adult body mass (capture mass range: 25.7-30.8 g) and has been found previously to provide a*d libitum* intake to Eastern Bluebirds (Siefferman, pers. comm.). Following the premature death of two infected birds that had suffered high mass loss (Balenger, unpubl. data), however, 60 g of mealworms were provided to the surviving infected birds for the last four days of the experiment (Figure 1). By this date, control birds had already been euthanized (see below), thus, the amount of food was only adjusted for birds infected with *M. gallisepticum* and not for the individuals inoculated with sterile media. Throughout the experiment, birds were also provided with water that included a soluble vitamin supplement (Wild Harvest Premium Multi-Drop for birds).

Figure 1. Timeline showing the critical timepoints and dates on which measurements and samples were taken for a) control and b) *M. gallisepticum* infected birds. (D: day)



The day before inoculation, mass and hemoglobin levels of each bird were measured (Figure 1). To measure hemoglobin, a sterile needle was used to puncture the

brachial wing vein, and a small drop of blood was collected directly into a capillary cuvette. Hemoglobin level (g/dL) was then determined using a HemoCue Hb 201+.

The following day, birds were inoculated with SP4 sterile culture media or a cultured *M. gallisepticum* field isolate collected from a House Finch in January 2007 following standard protocols (Farmer et al. 2005; Bonneaud et al. 2011; Balenger et al. 2015). Briefly, birds were infected with 20 µL of 1×10^4 to 1×10^6 color changing units of culture via ocular inoculation. Control and infected birds were maintained within the same room, separated by ZipWalls to limit any aerosolized spread of the pathogen. For the next 14 days, birds were monitored daily for signs of conjunctivitis.

Thirteen days following inoculation, mass and hemoglobin measures were collected again, as well as choanal swabs (Figure 1). Briefly, sterile culture swabs were inserted into the beak of each bird and gently swirled against the choanal cleft palate for 3 seconds. Swabs were placed at -20°C until DNA extraction was performed. The following day, birds were anesthetized with isoflourane and euthanized via rapid decapitation. At this time, the right eyes along with the surrounding conjunctival membrane were removed from the skull and stored in RNAlater at -80°C. All animal protocols and procedures were approved by the Institutional Care and Use Committee of the University of Mississippi (protocol #17-012) as well as state (0119183) and federal (MB13567C-1) collecting permits granted to S. L. Balenger.

DNA extraction

Conjunctiva and eye samples were placed in phosphate buffer solution (PBS) at 4°C for 24 hours prior to extraction to allow tissues to rehydrate. Using sterile forceps

and a sterile razor blade to mince the eye into small pieces, we homogenized the previously collected eye tissue. The tissue was placed into a sterile 5 mL tube with 360 µL of Qiagen ATL buffer and 200 µL of Qiagen proteinase K. A sterile pestle was used to grind the contents of the tube for 2-3 minutes. The mixture was then vortexed for approximately 10 seconds. This procedure of grinding and vortexing was repeated as necessary until the mixture resembled a "slurry". The mixture was then transferred to a 1.5 mL tube and ground further with a new sterile pestle and periodic vortexing for 5 second intervals. The samples were then placed at 56°C for 48 hours. A 40 µL aliquot of the homogenized tissue was extracted using a Qiagen DNeasy Blood and Tissue Kit and following the standard kit protocol.

Choanal swabs were extracted similar to Staley et al. (2018). Briefly, each swab was thawed and transferred to microcentrifuge tubes containing 200 µL of sterile water. Samples were incubated at 95°C for 10 minutes to burst cells, releasing their contents from the swabs into solution. Samples were placed on ice for 10 minutes and the swabs were removed from the tubes. DNA was extracted from samples using a Qiagen DNeasy Blood and Tissue Kit and following the standard kit protocol.

Quantitative PCR

We used quantitative PCR (qPCR) to determine the bacterial load of both the right eye samples and swabs from the choanal cleft palate. The DNA concentration of all samples was measured using BioTek's Epoch Microplate Spectrophotometer and Gen5 data analysis software. Conjunctival samples were diluted to 10 ng/µL. Because the DNA yields from some choanal swab extractions were too low to normalize, conjunctival DNA

concentrations ranged from 1-10 ng/µL. The results of our standard curve confirmed that these concentrations were sufficient for detection of low levels of *M. gallisepticum* (Figure 2). Reactions followed the TaqMan-based assay as described in Grodio et al. (2008). The genes that were amplified during the reaction were a single-copy Eastern Bluebird gene, *rag-1*, and a single-copy *M. gallisepticum* gene, *mgc2*. The use of the *rag-1* gene served as an internal control for the reaction as it 1) confirmed the presence of Bluebird DNA regardless of whether the samples amplified for the *mgc2* gene, and 2) could be used to normalize the relative amount of *M. gallisepticum* DNA in choanal swabs. Controls included water, *M. gallisepticum* DNA extracted from cells grown in culture, and Eastern Bluebird DNA from an individual negative for *M. gallisepticum*. Using Qiagen's QIAgility system, 15 µL of the pre-made master mixes were pipetted into each of the wells of a single Rotor-Gene Q 72-well rotor disc. Half of the wells contained the *rag-1* gene master mix and the other half contained the *mgc2* master mix. The QIAgility pipetted 5 µL of control or sample into the appropriate wells. All reactions were run on a single ring to avoid sample ring variation. Controls and samples were run in triplicate for each of the two primer pairs, a forward and reverse primer for r*ag-1* and *mgc2* (Grodio et al. 2008). The reaction was run on Qiagen's Rotor-Gene Q real-time PCR system. The reaction parameters were as follows: 95°C for 20 seconds followed by 45 cycles of 95°C for 3 seconds and 60°C for 30 seconds.

Standard Curve

To validate the qPCR assay, we made a standard curve using four tenfold serial dilutions of DNA from *M. gallisepticum* grown in cell culture media as well as Eastern Bluebird DNA. Starting with an original sample concentration of 10 ng/µL, 10 µL of

sample was added to 90 µL of water. This was repeated until reaching a final sample concentration of 0.001 ng/µL. Water was used as a no template control (NTC) in both reactions. Each of the Bluebird and *M. gallisepticum* DNA dilutions as well as the NTC was run in triplicate with each primer pair.

Analysis

Cycle quantification values (Cq) were determined using LinRegPCR. Cq values >40 were considered negative. Absolute quantity of *M. gallisepticum* in the eye plus conjunctiva tissue was calculated by interpolating Cq values into the standard curves. Because we did not obtain enough DNA from choanal swabs to standardize their reaction DNA concentrations, we could not use the standard curve to calculate the absolute number of *M. gallisepticum* present in this tissue. Instead, we calculated the relative amount of *M. gallisepticum* in each sample by comparing the mean *rag-1* and *mgc2* Cq values of each sample's set of triplicates using the equation $\bar{x}$ *rag-1*Cq/ $\bar{x}$ *mgc2*Cq. These values were used to compare the relative quantity of *M. gallisepticum* to individual mass, mass change, body condition, and hemoglobin levels. Mass change was calculated as the difference in mass between the day before the experiment and 13 days following infection. Body condition was calculated as mass 13 days following inoculation relative to wing length on the day of capture. This minimizes the effects of captivity on wing wear.

We used Kruskal-Wallis tests to statistically determine if measurements of individual mass, mass change, body condition, and hemoglobin levels of infected birds were significantly different than those of control birds. Spearman rank order correlations

of individuals inoculated with *M. gallisepticum* were calculated with respect to individual mass, mass change, body condition, and hemoglobin levels to determine if any of these measurements was significantly correlated with pathogen load.

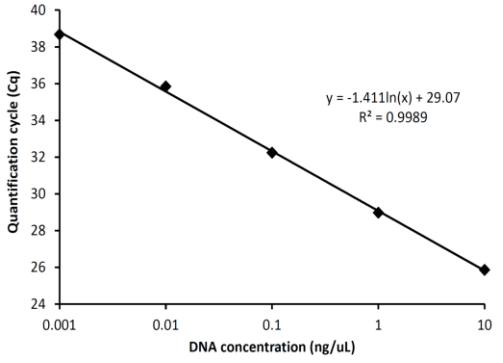
Results

Of the 11 birds infected with *M. gallisepticum* in this experiment, two died before the experiment was completed. Before dying, one of the birds lost 33% of mass by day 8 and the other lost 30% of mass by day 9. Thus, the final sample sizes for control and infected groups were 12 and 9, respectively.

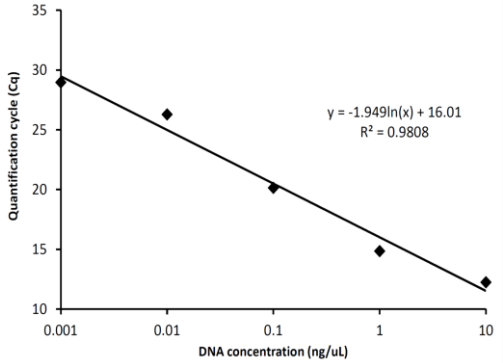
The qPCR-based *rag-1* and *mgc2* standard curves are shown in Figure 2. These curves were ultimately not used to quantify the amount of *M. gallisepticum* in the conjunctiva because the *mgc2* locus did not amplify from any of the DNA extracted from tissue associated with the eye. However, of the nine experimentally infected birds, six tested positive for *M. gallisepticum* in the choanal cleft palate 13 days following inoculation.

Figure 2. Standard curves of a) *rag-1* and b) *mgc2*. Line equations and regression coefficients are provided for each dilution series.

a)



b)



Mass, mass change, and body condition did not significantly differ 13 days following inoculation between infected and uninfected birds. However, birds that were infected were found to have lower levels of circulating hemoglobin than control birds (Table 1, Figure 3). When examining only those birds infected with *M. gallisepticum*, we found that birds with a greater pathogen load 13 days following inoculation lost the most mass over the course of the infection (Table 2). No other measures of condition or pathology were significantly associated with *M. gallisepticum* load (Table 2).

Table 1. Results of Kruskal-Wallis tests assessing whether inoculation with *M. gallisepticum* affects condition or health compared to control birds that remained uninfected throughout the experiment. Significant *p*-values are in bold.

	degrees of freedom	χ^2	*p-value*
Body condition	1	2.020	0.155
Mass change (g)	1	1.293	0.256
Hemoglobin (g/dL)	1	3.824	**0.050**
Mass (g)	1	3.416	0.065

Figure 3. Mean a) body condition, b) mass change, c) hemoglobin level, and d) individual mass of birds inoculated with *M. gallisepticum* and control birds with standard error bars. An asterisk identifies a significant difference observed between the control and *M. gallisepticum* inoculated groups.

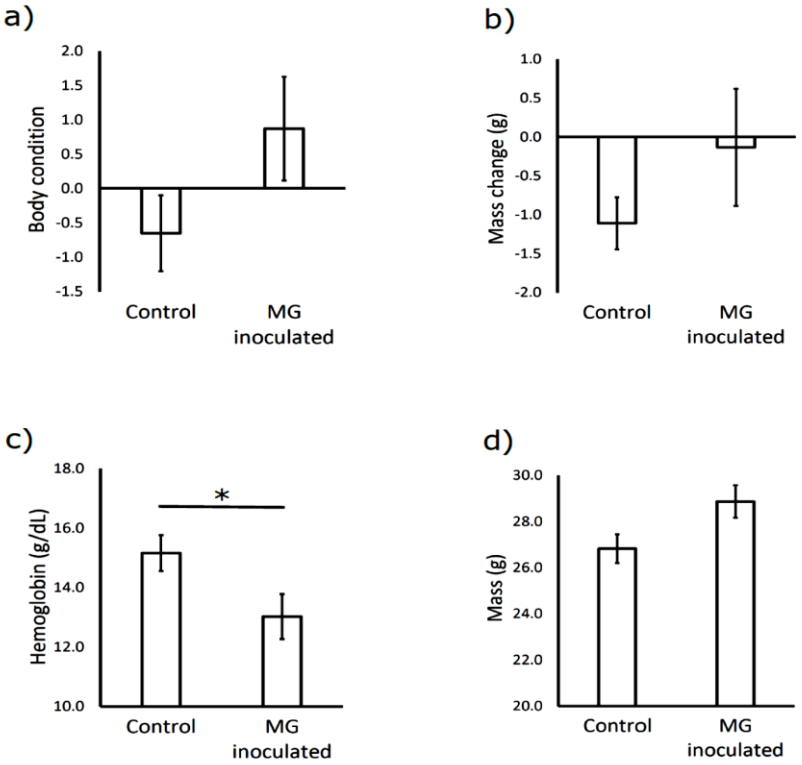


Table 2. Spearman rank order correlations of individuals inoculated with *M. gallisepticum*. Each column shows the effect size (*rho*), associated *p*-value, and sample size (n). Significant *p*-values are in bold.

		Body condition	Mass change (g)	Hemoglobin (g/dL)	Mass (g)
Pathogen load	*rho*	-0.034	-0.712	-0.475	-0.223
	p-value	0.931	**0.031**	0.1967	0.552
	n	9	9	9	9

Discussion

Previous studies investigating the cost of *M. gallisepticum* infection upon songbird hosts have primarily relied on the presence of visual scoring of conjunctivitis symptoms to evaluate the severity of disease state, host resistance, and pathogen virulence (Farmer et al. 2005; Balenger et al. 2015). Here we showed that relying solely on this metric is inadequate for Eastern Bluebirds, which suffer from costly pathogenesis when infected with *M. gallisepticum* but never develop symptoms of conjunctivitis. Unlike in House Finches, *M. gallisepticum* does not localize to the eye conjunctiva of Eastern Bluebirds following infection; this is likely the factor behind the lack of ocular swelling in response to infection. We did find, however, that *M. gallisepticum* continues to persist within the choanal cleft palate for at least 13 days in infected Eastern Bluebirds, thus, this species is capable of functioning as a natural host in the wild. Infection *per se* was a predictor of hemoglobin levels, such that infected birds had lower levels of circulating hemoglobin. Using relative pathogen load data among experimentally infected birds, we were able to investigate whether the amount of pathogen remaining 13 days after inoculation, a metric of resistance, was related to costs of pathogenesis or immunity. We found that relative pathogen load was a determinant of the amount of mass lost over the course of the experiment. Essentially, those birds with the highest pathogen load had also lost the most mass. Evidence from similar studies of *M. gallisepticum* infected House Finches supports the interpretation that birds' observed mass loss is not occurring

as cost to the host for mounting an immune response, but instead these birds are suffering the most from pathogenesis (Bonneaud et al. 2012).

To investigate the cost of infection, as opposed to the cost of immunity, on Eastern Bluebird health, we examined host levels of circulating hemoglobin. We found that experimentally infected Eastern Bluebirds had significantly lower hemoglobin levels, suggesting that *M. gallisepticum* causes anemia in this host species. Many members of the *Mycoplasma* genus are hemotropic; adhering to, colonizing, and growing upon host erythrocytes (Messick 2004). Commonly referred to as haemoplasmas, hemotropic mycoplasmas are red blood cell pathogens that generate host infectious anemia (Messick 2004). Messick et al. (2002) identified the *pneunomiae* clade of *Mycoplasma* (which includes *M. gallisepticum*) as being closely related to the monophyletic group of haemoplasmas. In fact, although it is relatively rare, *M. pneumoniae* is known to cause hemolytic anemia in humans (Waites & Talkington 2004). Confirming that *M. gallisepticum* is the underlying cause of anemia in infected Eastern Bluebirds should therefore be pursued in future studies.

Somewhat surprisingly, we found that experimentally infected Eastern Bluebirds did not have *M. gallisepticum* present in their eye conjunctiva on day 14. DNA collected from the choanal cleft palate, however, identified the presence of *M. gallisepticum* in the palate of six of the nine surviving infected birds 13 days after the initial ocular inoculation.

Grodio et al. (2008) proposed that, at least for House Finches, bacterial load correlates with development and severity of conjunctivitis. In the current study, the absence of *M. gallisepticum* in the eye conjunctiva of ocularly infected birds is a likely

explanation for why Eastern Bluebirds do not develop conjunctivitis. It is possible that the conditions encountered by *M. gallisepticum* in the conjunctival lining surrounding the eyes in Eastern Bluebirds differ from that of House finches in ways that make it inhospitable to *M. gallisepticum*.

During this experiment, two infected birds died due to rapid and dramatic mass loss (Balenger, unpubl. data), one on day 8 and one on day 9. In response to this mortality, the amount of food the birds were receiving was doubled to prevent any further deaths. House Finches rarely, if ever, die in captivity in response to infection with *M. gallisepticum* (Bonneaud et al. 2011), and the birds used in the current study had all increased in mass between capture in the wild and the start of the experiment on the same diet they were receiving when mortality occurred (Balenger, unpubl. data). Even after doubling the mass of food available to experimental birds 10 days post-infection, we found that variation in mass change among infected birds still was related to *M. gallisepticum* load. Eastern Bluebirds harboring the most *M. gallisepticum* 13 days after inoculation lost the most mass over the course of the infection. The change to food being administered *ad libitum* on day 10 is likely to have affected the results of our investigation of the physiological costs associated with *M. gallisepticum* infection. Control birds did not receive the increased amount of food because they had already been euthanized at this time. It is possible that if all birds had remained this feeding schedule for the entirety of the study then infected birds would have increased their caloric uptake and therefore have more resources to put towards responding immunologically. It would be valuable to explore dietary manipulations in the future to determine how calories available interact with immunity. Infection with *M. gallisepticum* clearly increases

energetic costs to Eastern Bluebirds and survival rates and optimal physiological strategies of infected hosts will likely vary depending on availability of resources.

According to a long-term database (ebird.com) compiled by the Cornell Lab of Ornithology, Eastern Bluebird abundance during breeding and non-breeding seasons have been declining over large portions of their range between 2007-2016 (Figure 4). It is possible that *M. gallisepticum* infection could be related to the documented decreases in abundance. Because Eastern Bluebirds are still commonly found, these decreases in abundance can go unnoticed by the general population. It is important to take these data into consideration and investigate the possibility of *M. gallisepticum* infection being a factor in population size reductions. The same notion can apply for other novel *M. gallisepticum* host species experiencing similar decreases in abundance.

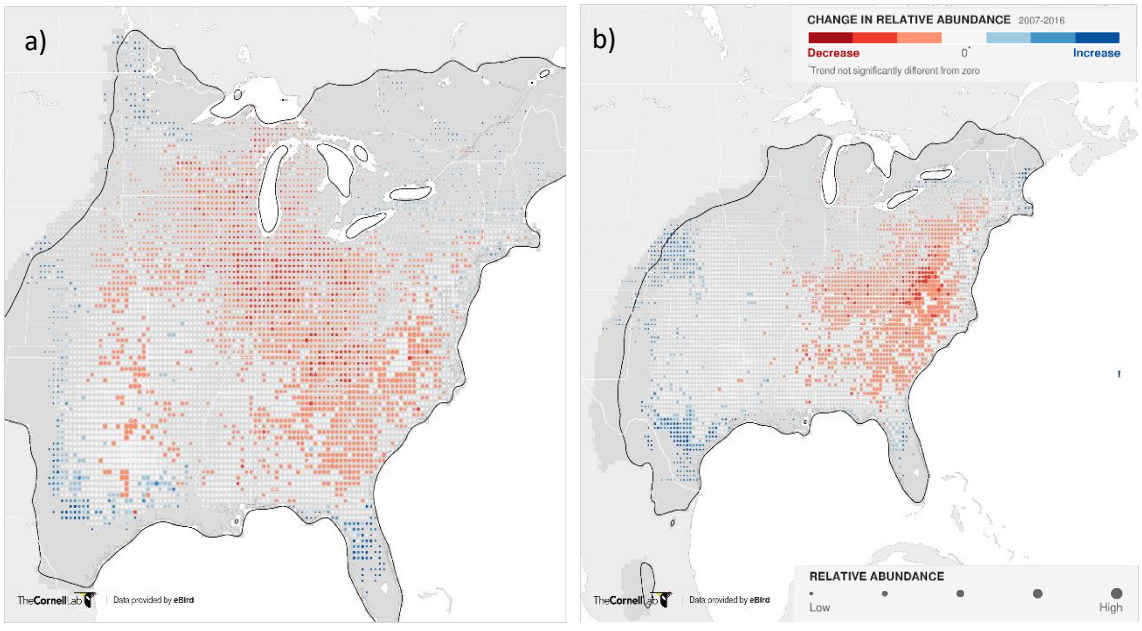


Figure 4. Map of changes in relative abundance of Eastern Bluebirds from years 2007-2016 during a) breeding season months (May 31 - Jul 6) and b) non-breeding season months (Nov 30 - Dec 28). Image provided by eBird (www.ebird.org) and created [November 2018].

Although, at the surface, Eastern Bluebirds seem to be asymptomatic hosts of *M. gallisepticum*, this study showed that they do suffer from physiological costs when infected. We hypothesize that this could be the case with other avian species that are currently viewed as carriers or reservoirs of *M. gallisepticum*. In the past, non-conjunctivitis-developing host species of *M. gallisepticum* were thought to suffer little to no cost of infection. For Eastern Bluebirds, this is clearly untrue. It is possible that there are other host species harboring the pathogen that do not develop conjunctivitis but also suffer from negative health consequences. Going forward, research should aim to determine if *M. gallisepticum* is having significant negative effects on the health of hosts. These relationships can give insight into how different host species are responding to infection in terms of coping strategies such as tolerance and resistance and whether *M. gallisepticum* is influencing population and community dynamics in ways previously unrecognized.

This study contributes to the growing knowledge of *M. gallisepticum* infection in Eastern Bluebirds. Our results show that Eastern Bluebirds are experiencing negative health effects after infection. Although these birds do not suffer from the consequences of conjunctival inflammation, they are likely facing physiological costs of infection.

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